ADDENDUM TO ANNUAL PROGRESS REPORT

INJURY OF THE SKIN

September 1, 1963 to February 29, 1964

By: University of Illinois
Chicago, Illinois

To: Office of Naval Research
Department of the Navy
Physiology Branch (Code 441)
Washington 25, D. C.

Grant No. NR102-628 Nonr00022-64

Principal Investigator

Sol Roy Rosenthal, M.D., Ph.D.

In Cooperation with

George Crouse
Wilma A. Spurrier

INTRODUCTION

This report is to supplement the report to the ONR of May 1, 1962 - May 1, 1963. The contents of this report was given in part at the Federation for Biological Societies Meetings in April 1964.

INTRAVENOUS LETHALITY OF HUMAN SKIN HOMOGENATES IN MICE

1. Lethality and Thromboplastin.

Lethal fractions were isolated from diffusates of burned rat skin using an air pocket technique (1) as well as from extracts of rat skin (2). At the Federation Meetings last year (3) a procedure was presented for isolating from normal human skin an homogenate which is lethal in mice. Data available at that time indicated that the lethal activity in homogenates could not be attributed to its clot accelerating properties. It is the purpose of this report to present additional data obtained in this regard. This information confirms earlier observations and establishes that thromboplastin, although probably present in the homogenates, is apparently not the major lethal factor.

The animals used to test the lethality of homogenates were 18 to 20 gram Swiss mice. They were injected intravenously into the lateral tail vein with 0.2 ml volumes. To determine lowest lethal levels, concentrations were varied by diluting the homogenates with saline. A dilution which killed 40% of the injected animals was considered to be lethal.

Flow Sheet 1 shows the method now used to obtain these homogenates. The tissues were taken from both surgical and coroner's cases. After low speed centrifugation, 3000RPM (3T), the first homogenate separates

into three major fractions. The fatty float and large particle fractions are discarded, and the small particle fraction (hereafter called the 3T homogenate) is subjected to various experimental procedures in an attempt to elucidate the nature of its lethal factor.

Table 1 gives the lethality in mice of these fractions. It may be seen that the hot (extractions made in 96°C saline) 3T homogenates are more consistantly lethal than are the cold (extractions made in 22°C saline). It is apparent that it requires 27% less of the hot 3T homogenate to induce death in the animal. The fact that hot and cold homogenates are not equally lethal is in itself an observation inconsistant with the assumption that thromboplastin is responsible for the lethal effects (4). If it were the only factor involved in lethality, one would expect the hot 3T homogenate to be slightly less lethal, since thromboplastin is partially heat labile.

Our research is primarily directed toward the study of the so-called "burn toxin," and since this toxin is considered to be a degradation product resulting from injury to the cell, most of this discussion will center about the hot 3T homogenate.

These fractions were subjected to several procedures that affect to varying degrees the clot accelerating activity of thromboplastin. It was determined whether or not procedures which are known to inhibit thromboplastin would also destroy or reduce the level of lethality due to the skin homogenates. The results of these experiments are given in Table 2. Incubation, suspension in sodium hydroxide, and suspension in sodium heparin cause only a slight decrease in the lethal levels in mice.

This is what one would expect if thromboplastin is present in the homogenates in a form capable of accelerating clot formation or increasing blood viscosity. Refluxing in 80% ethanol for one and one-half hours, which completely destroys the thromboplastic and lethal activity in brain thromboplastin from several species, failed to bring about a reduction in the lethality levels due to the skin homogenates. Likewise, extraction in ether causes an increase in the lethality of the homogenates which would be expected if thromboplastin is present along with an inhibitor that is soluble in ether. On the other hand, ether may remove the lipoid component of thromboplastin which should reduce its activity. That an inhibitor may be present is seen from Graph 1 which shows the effect of dilutions of the homogenate on the clot acceleration activity of commercial rabbit brain thromboplastin.

Some of the homogenates were used to replace commercial thromboplastin in one-stage prothrombin tests. The data from one series of tests is given in Table 3. Obviously under the conditions of the test the homogenates fail to accelerate the clotting of normal plasma. Commercial rabbit brain thromboplastin in these tests coagulated normal plasma in 12 seconds. Assuming the activity of commercial thromboplastin to be 100% (if such is valid) and taking this as a base line, the clot accelerating activity in the homogenate is less than 2%. In Hicks-Pitney (5) modification of the thromboplastin generation test, at tissue concentrations of 0.86 mg/ml, thromboplastin was generated to about 13% in excess of that measured by the direct one-stage prothrombin test. It is important to note at this point that 0.225 mu/ml of sodium heparin is sufficient to completely inhibit the clot acceleration ability of the homogenates in the Hicks-Pitney test.

In one experiment 18 to 20 gram animals were injected intravenously with 200 units of sodium heparin. This initial injection was followed within 5 minutes by a second injection with tissue homogenate. Under these conditions and at tissue concentrations of 4.1 mg/ml, heparin failed to decrease the lethal level due to the homogenate. In the case of commercial thromboplastin at a concentration of 12.5 mg/ml heparin completely destroyed the lethal effect. However, if 500 units of heparin was injected prior to the skin homogenate, the lethal level was lowered from 0.043 mg/ml gram animal to 0.086 mg/gram animal. In either case, however, 10^3 more heparin was injected than was required to inhibit the in vitro clot acceleration properties of the homogenates. During the course of these studies, it was noticed that if the 3T homogenates were treated at room temperatures for 10 minutes in 0.1 N sodium hydroxide, centrifuged at 34.8 x 1000 G, precipitated in cold ethanol, suspended in water, dialized for 24 hours against saline and recentrifuged at 34.8 x 1000 G for 30 minutes a clear supernatant fluid could be obtained which was lethal when injected intravenously into mice. Table 4 gives some of the preliminary information on this fraction. All fractions were also ninhydrin negative and orcinol and diphenylamine positive. Ultraviolet spectra showed a plateau between 260 and 230 mu with a center of minimum absorption at 250 mu and a continuously increasing end-absorption in the low wave lengths. This spectrum is like, but not identical with, that reported by Chargaff for thromboplastic protein. The center of maximum absorption at 227 mu could not be measured as found for thromboplastin, and the plateau obtained to include 280 mu.

It is not known if these variations in the spectra are due to the manner in which the supernatants were treated, to contaminating substances, or are in fact real. This fraction also failed to accelerate clot formation in normal plasma. Further studies on these preparations are now in progress.

TO SUMMARIZE THEN: Homogenates prepared in 96°C saline are more consistantly lethal then are homogenates prepared in 22°C saline, and about 27% by weight more of the cold homogenates is required to produce death in mice. In general the treatments to which the homogenates were subjected gave results consistant with the idea that clot accelerating properties but with lethal capabilities can be isolated also indicates that thromboplastin is not the lethal compound in the homogenates.

REFERENCES

1. On an In Vivo Method of Collection of Diffusates from Skin: Thermal and Radiation Injury, S. R. Rosenthal, F. R. Hunter, F. J. Finamore and I. N. Roman, Arch. Int. pharmacodyn, 126, 43, 1960.

2. Substances Released from the Skin Following Thermal Injury, S. R. Rosenthal, Surgery, 46, 932, 1959.

3. Intravenous Lethality of Human Skin Homogenates In Mice: Lethality and Thromboplastin, S. R. Rosenthal, G. T. Crouse and W. A. Spurrier. Paper given at meeting of Federation Amer. Society for Exper. Biology, April 14, 1964.

4. Toxic Thromboplastic Extracts of Skin, C. L. Fox, Ian A. Holder, Lucinda L. Melin, JAMA 187, 655, 1964.

5. Thromboplastin Generation Test, N. D. Hicks, W. R. Pitney, Brit. J. Haem., 3, 277, 1957.

FLOW SHEET I

EXTRACTION OF "BURN TOXIN"
FROM BURNED NORMAL HUMAN SKIN

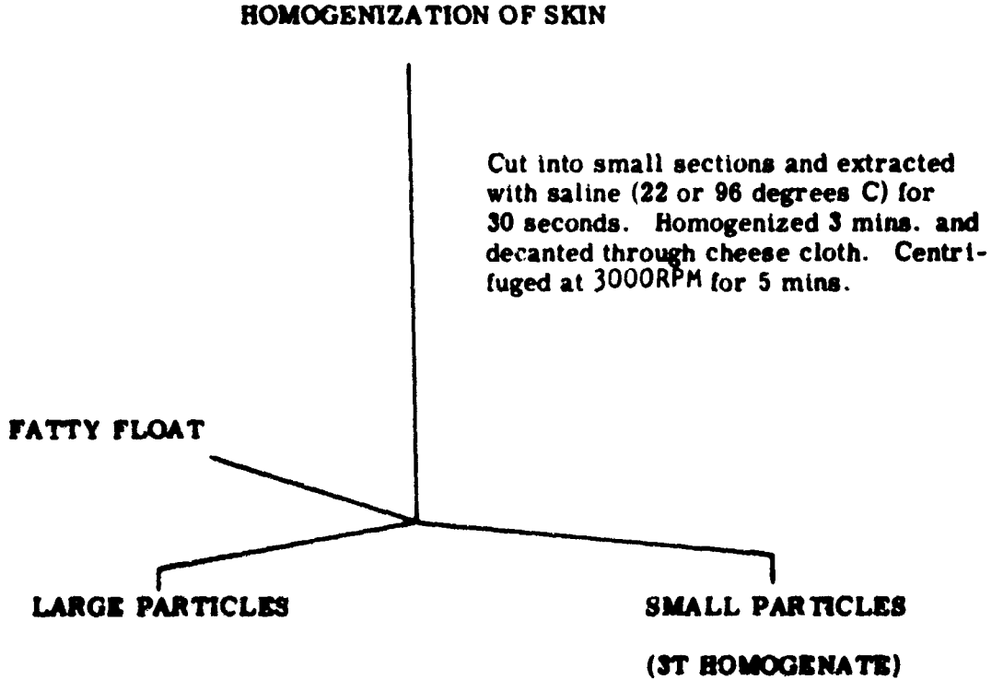

TABLE I

LETHALITY OF 3T HOMOGENATES
(EXTRACTED IN 22°C AND IN 96°C SALINE)*

Fraction	Number of Experiments	% of Animal Death	% Experiments Lethal 40% Level	MG/G Animal Lethal
Cold 3T Homogenate	5 (3 - L, 2 - NL)	48%	66.0	0.123
Hot 3T Homogenate	5 (5 - L, 0 - NL)	92%	100.0	0.096

* Both cold and hot homogenates were from same skin sample and injected in 0.2 ml amounts intravenously into 18 to 20 gram animals.

Note:
Mg/gram Animal Lethal Range: Cold = 1.2 - 2.8 and Hot = 0.4 - 2.8

TABLE 2

EFFECT OF VARIOUS PROCEDURES ON THE LETHALITY OF 3T HOMOGENATES

Treatment	Before Treatment		After Treatment	
	MG/G Animal Lethal	% Death	MG/G Animal Lethal	% Death
Incubation 56°C/30 Mins.	0.128	94	0.128	75
Incubation 37°C/7 Days	0.064	94	0.120	100
Dialized Homogenate Incubation Room Temp./1 Hr. In 0.10 N NaOH	0.004	40	0.006	100
Dialized Homogenate Extraction - Ether 4 x at R.T.	0.004	40	0.003	75
Dialized Homogenate Extraction - Ethanol 4 x at R.T.	0.004	40	0.010	80
Dialized Homogenate Extraction - Ethanol/Ether 3:1 at R.T.	0.004	40	0.011	75
Suspensions in 5% Na-Heparin	0.064	92	0.142	91
Refluxing 1.5 Hours in 80% Ethanol	0.160	100	0.160	100

GRAPH I

Inhibition of Commertial Rabbit Brain Thromboplastin by Burned Skin (Human) Homogenate

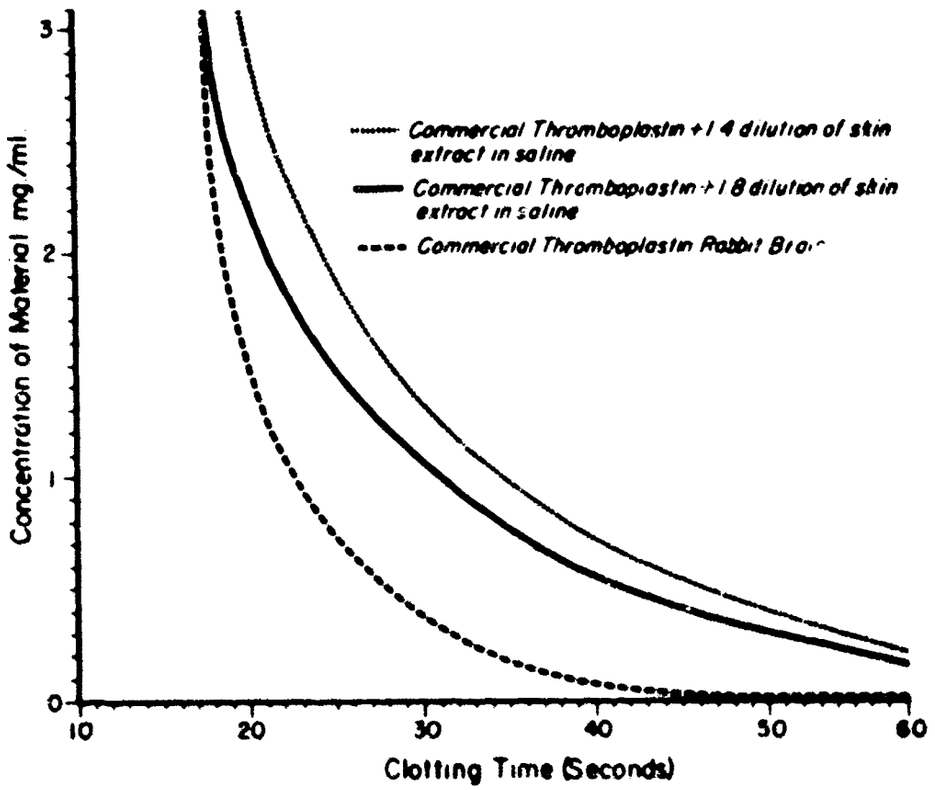

TABLE 3

PLASMA COAGULATION ACTIVITY
OF 3T HOMOGENATES

Sample MG. ML Dry Weight	Seconds Prothrombin Time
CC Homogenate Diluted in Saline 17.2	720
8.6	780
4.3	1200
2.65	1080
1.33	1680
0.86	∝

Normal Plasma Generation Test - 16 seconds
Normal Plasma One-Stage Test - 12 seconds
Ca ++ Clot -342 seconds

TABLE 4

PARTICLE FREE FRACTION PROPERTIES

Experiment	Grams Starting Material	M/G Animal Lethal	Dry Weight	pH	Protein	Light Scatter
1	25	0.460	8.3	6.5	5.2	5%
2	25	0.242	8.8	6.5	5.5	10%
3	25	Toxic	-	7.0	-	-
4	25	Lethal	-	6.5	-	5%

UNIVERSITY OF ILLINOIS
CHICAGO 12, ILLINOIS

March 1, 1965

Department of Commerce
Office of Technical Services
Acquisitions Branch
Washington 25, D. C.

Gentlemen:

There has been a typographical error (omission of four or five lines in the summary) in my paper:

ADDENDUM TO ANNUAL PROGRESS REPORT: INJURY OF THE SKIN.

I am attaching pages 5 and 6 to replace page 5 in the original report. I am sorry for this omission.

Sincerely yours,

Sol Roy Rosenthal

Sol Roy Rosenthal, M.D., Ph.D.
Director

SRR:FW
Enclosures

It is not known if these variations in the spectra are due to the manner in which our supernatants were treated, to contaminating substances, or are in fact real. This fraction also failed to accelerate clot formation in normal plasma. Further studies on these preparations are now in progress.

TO SUMMARIZE THEN: Homogenates prepared in 96°C saline are more consistantly lethal than are homogenates prepared in 22°C saline, and about 27% by weight more of the cold homogenate is required to produce death in mice. In general the treatments to which the homogenates were subjected gave results consistant with the idea that clot accelerating compounds are present or may be generated from the homogenates to a low degree only. However, the fact that refluxing in 80% ethanol for one and one-half hours and suspension in heparin failed to eliminate the lethal effect, speaks for the possibility that very little if any of the lethal effect of the homogenates can be attributed to these substances. Finally, the fact that a particle free fraction without clot accelerating properties but with lethal capabilities can be isolated also indicates that thromboplastin is not the lethal compound in the homogenates.

REFERENCES

1. Rosenthal, S. R., F. R. Hunter, F. J. Finamore and I. N. Roman. On an In Vivo Method of Collection of Diffusates from Skin: Thermal and Radiation Injury. Arch. Int. Pharmacodyn, 126, 43, 1960.

2. Rosenthal, S. R. Substances Released from the Skin Following Thermal Injury. Surgery, 46, 932, 1959.

3. Rosenthal, S. R., G. T. Crouse and W. A. Spurrier. Intravenous Lethality of Human Skin Homogenates In Mice: Lethality and Thromboplastin. Paper given at meeting of Federation Amer. Society for Exper. Biology, April 14, 1964.

4. Fox, C. L., Ian A. Holder, Lucinda L. Malin. Toxic Thromboplastic Extracts of Skin. JAMA 187, 655, 1964.

5. Hicks, N. D., W. R. Pitney. Thromboplastin Generation Test. Brit. J. Haem., 3, 277, 1957.